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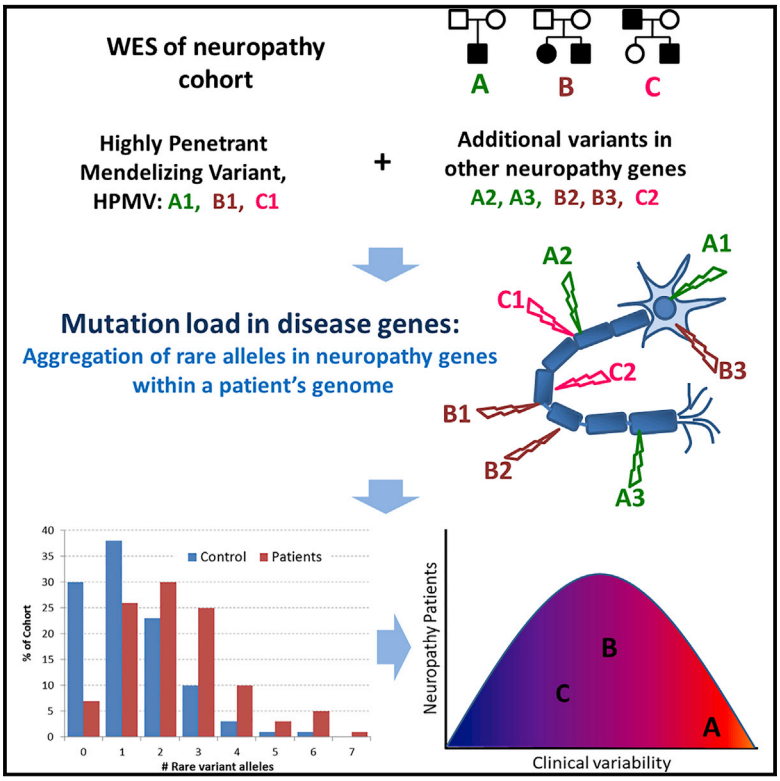
Exome Sequence Analysis Suggests that Genetic Burden Contributes to Phenotypic Variability and Complex Neuropathy

Graphical Abstract



Highlights

- WES of a neuropathy cohort identifies causal variants in ~45% of patients
- Three candidate disease genes associated with peripheral neuropathy are proposed
- Evidence for genetic mutation burden is found in two independent cohorts
- Variant combinatorial effects may contribute to clinical variability and expressivity

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In Brief

Peripheral neuropathy is a clinically variable and genetically heterogeneous disease. In a cohort of patients, Gonzaga-Jauregui et al. have identified causative variants in ~45% of the families studied, proposed candidate disease genes for an additional three families, and recognized a significant mutation burden in patients versus controls that likely contributes to phenotypic variability.

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Exome Sequence Analysis Suggests that Genetic Burden Contributes to Phenotypic Variability and Complex Neuropathy

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SUMMARY

Charcot-Marie-Tooth (CMT) disease is a clinically and genetically heterogeneous distal symmetric polyneuropathy. Whole-exome sequencing (WES) of 40 individuals from 37 unrelated families with CMT-like peripheral neuropathy refractory to molecular diagnosis identified apparent causal mutations in ~45% (17/37) of families. Three candidate disease genes are proposed, supported by a combination of genetic and in vivo studies. Aggregate analysis of mutation data revealed a significantly increased number of rare variants across 58 neuropathy-associated genes in subjects versus controls, confirmed in a second ethnically discrete neuropathy cohort, suggesting that mutation burden potentially contributes to phenotypic variability. Neuropathy genes shown to have highly penetrant Mendelizing variants (HPMVs) and implicated by burden in families were shown to interact genetically in a zebrafish assay exacerbating the phenotype established by the suppression of single genes. Our findings suggest that the combinatorial effect of rare variants contributes to disease burden and variable expressivity.

INTRODUCTION

Charcot-Marie-Tooth (CMT) disease, first described clinically in 1886 (Charcot and Marie, 1886; Tooth, 1886), is a common hereditary peripheral neuropathy with an estimated prevalence of one in 1,200 (Braathen, 2012) to one in 2,500 (Skre, 1974) individuals. The disease is characterized by distal symmetric polyneuropathy (DSP) with progressive muscle weakness and atrophy, as well as sensory loss. Two major clinical

types are distinguished by electrophysiologic and neuropathologic studies and the type of cells (glia or neurons) primarily affected. CMT1 affects the glia-forming Schwann cells and presents with nerve conduction velocities (NCV) of <38 m/s; CMT2 affects the axons of neurons and usually presents with NCVs of >38 m/s or slightly reduced motor NCVs but with diminished amplitudes. Other forms of CMT with additional clinical features have been described, including an intermediate form with overlapping demyelinating and axonal CMT features (Nicholson and Myers, 2006) and one in which CMT occurs in conjunction with glomerulonephritis (Boyer et al., 2011).

The observed inheritance patterns include autosomal dominant, autosomal recessive, and X-linked (dominant and recessive) forms (Allan, 1939; Rossor et al., 2012). Nevertheless, most patients present with apparent sporadic disease, attributable partially to the extreme clinical variability and age-dependent penetrance of the phenotype. New mutation, however, is often the cause of sporadic CMT, with the de novo CMT1A duplication of 17p11.2 being responsible for 76%–90% of sporadic cases (Raeymaekers et al., 1991; Lupski et al., 1991; Hoogendijk et al., 1992; Nelis et al., 1996). Locus-specific screening for mutations in known CMT genes concludes a molecular diagnosis for approximately 70%–80% of patients (Szigeti and Lupski, 2009; DiVincenzo et al., 2014). More than 40 genes are known to be causative, but it has been estimated that 30–50 "CMT genes" remain to be discovered (Braathen, 2012; Timmerman et al., 2014).

CMT1A (MIM #118220) is caused by a recurrent 1.4-Mb duplication that encompasses the dosage-sensitive myelin gene *PMP22* (Lupski et al., 1991; Hoogendijk et al., 1992; Patel et al., 1992; Lupski et al., 1992), an essential component of compact peripheral nervous system (PNS) myelin (Li et al., 2013). The reciprocal deletion of the identical 17p11.2 region causes hereditary neuropathy with liability to pressure palsies (HNPP) (MIM #162500) (Chance et al., 1993, 1994). A recent study of 17,000 patients with neuropathy established a molecular diagnosis in 18.5% of these; ~80% of molecular diagnoses were either duplication or deletion of *PMP22* (DiVincenzo et al., 2014). Point mutations and indels in *PMP22* have also been found in patients with CMT1A or HNPP without duplication or deletion (Roa et al., 1993a; Nicholson et al., 1994), and in the more severe early-onset phenotype of hypertrophic neuropathy of Dejerine-Sottas (MIM #145900) (Dejerine and Sottas, 1893; Roa et al., 1993a, 1993b; Li et al., 2013). Additionally, non-recurrent and complex rearrangements can account for the missing heritability in CMT1A and HNPP, including upstream copy-number variants (CNVs) that do not include *PMP22* coding sequence (Zhang et al., 2010; Weterman et al., 2010).

The second most common form of CMT is CMTX1 (MIM #302800) caused primarily by point mutations that occur in almost every amino acid of *GJB1/connexin32* (Kleopa and Scherer, 2006; Scherer and Kleopa, 2012); gene deletions have also been described (Gonzaga-Jauregui et al., 2010). *GJB1* encodes a gap junction protein involved in the formation of connexon hemichannels that facilitate the communication and exchange of ions and other small molecules between Schwann cells and axons (Scherer and Kleopa, 2012).

The third most common cause of CMT, and the most common form of CMT2, are heterozygous mutations in *MFN2* (CMT2A; MIM #609260) (Ben Othmane et al., 1993; Züchner et al., 2004; Verhoeven et al., 2006), essential for mitochondrial fusion and function (Kijima et al., 2005) and maintenance of mitochondrial morphology. Mutations in *MFN2* lead to mitochondrial dysfunction due to mtDNA depletion (Vielhaber et al., 2013). Mutations in *GDAP1* cause a recessive form of CMT, which can be either demyelinating (CMT4A; MIM #214400) (Cuesta et al., 2002), axonal (CMT2K; MIM #607831) (Nelis et al., 2002) or intermediate (CMTRIA; MIM #608340) (Senderek et al., 2003) and have been reported to affect mitochondrial fission in Schwann cells and neurons (Niemann et al., 2005).

Known CMT genes encode proteins that span a wide range of functions, from GTPases (*RAB7*, *DNM2*), lipid phosphatases (*FIG4*, *MTMR2*), to structural myelin proteins (*MPZ*, *PMP22*) and gap junction channel components (*GJB1*). Cellular functions include myelin assembly (*PMP22*, *MPZ*, *PRX*, *Cx32*), membrane and endocytic trafficking (*MTMR2*, *SBF2*, *FIG4*, *SH3TC2*) and mitochondrial dynamics (*MFN2*, *GDAP1*) (Niemann et al., 2005; Azzedine et al., 2012). Another predominant contributing gene group is that of aminoacyl-tRNA synthetases, an essential class of enzymes that ligate amino acids onto cognate tRNA molecules (reviewed in Wallen and Antonellis, 2013).

Other complex forms of CMT2 (e.g., spinocerebellar ataxia with axonal neuropathy, SCAN1) have been associated with mutations in *TDP1*, important for DNA single-strand break repair (SSBR) (McKinnon and Caldecott, 2007; Caldecott, 2008). Mutations in *SETX*, a helicase involved in transcriptional termination and RNA maturation, cause recessive ataxia ocular motor apraxia type 2 (AOA2; MIM #606002) (Moreira et al., 2004) possibly due to transcriptional/translational defects (Anheim et al., 2012), also disturbing DNA SSBR (Caldecott, 2008). *SETX* mutations have been associated with familial amyotrophic lateral sclerosis (ALS), susceptibility that recently was also associated with heterozygous *FIG4* mutation carrier states (Chow et al., 2009).

Substantial genetic and clinical heterogeneity of CMT neuropathy makes it challenging for molecular diagnosis by single gene and gene panel testing; the diagnostic utility of genome-wide sequencing approaches has been demonstrated (Lupski et al., 2010; Montenegro et al., 2011; Choi et al., 2012; Lupski et al., 2013). We performed whole-exome sequencing (WES) in a cohort of 40 patients with peripheral neuropathy from 37 unrelated families in whom extensive genetic evaluation had failed to identify a causative mutation or establish a molecular diagnosis (Tables 1 and S1). Analysis of WES data was performed in two stages: a first-pass analysis that focused on known or novel variants in known CMT and related neuropathy genes, and a second stage analysis to search for rare variants in likely novel candidate genes (Figure S1). Our rare variant analyses revealed potential neuropathy candidate "disease genes." Surprisingly, we uncovered evidence for a mutational burden in affected individuals versus a large sample of unrelated control individuals. We show experimentally that genetic interactions implicated by burden contribute to phenotypic variability and potentially to susceptibility to common neuropathies beyond the well-characterized Mendelian forms.

Table 1. Personal Exome Findings in Neuropathy Genes Reveal Highly Penetrant Mendelizing Variants (HPMVs) Driving Disease, Additional Genes Harboring Rare Variants, and Diagnosis Based On Molecular Findings

BAB #	Clinical Dx	Main Mutation(s) Identified (HPMV)	Additional Genes with Rare Variants	Molecular Dx [MIM #]
Known genes, known variants				
668	CMT2	*MFN2* (p.W740S)[b]	*MED25, IKBKAP, TRPV4*	CMT2A2 [609260]
710	CMT2	*MED25* (p.A335V)[b]; (p.P656T)[c]	*ATP7A, SH3TC2*	CMT2B2 [605589]
1405	CMT1	*MFN2* (p.V244M)[b] [de novo]	*WNK1*	CMT2A2 [609260]
1564	CMT2	*MFN2* (p.V244M)[b] [de novo]	*HSPB1, NARS2, TARS*	CMT2A2 [609260]
3656 (T)	CMT2	*AARS* (p.R329H)[b]	*SFB2*	CMT2N [613287]
3662 (T)	CMT2	*MFN2* (p.C281S)[b]	*SBF2, INF2, KIF1B*	CMT2A2 [609260]
3663 (T)	CMT1	*MPZ* (p.I135L)[b]	-	CMT1B [118200]
Known genes, novel variants				
1080	CMT	*MFN2* (p.R649P)[c]	*GDAP1, DNMT1*	CMT2A2 [609260]
1280	CMT2	*ARHGEF10* (p.G132S)[c]	*CARS2*	AD slowed nerve conduction velocity [608236]
1500	Dejerine Sottas	*SH3TC2* (p.K274X)[c] [hmz]	*DNM2*	CMT4C [601596]
1955	CMT2	*AIFM1* (p.R463I)[c]	*AARS, KIF1B, WNK1*	Cowchock syndrome [310490]
3646	CMT intermediate	*YARS* (p.E196Q)[c]	*DNMT1, MARS*	CMTDIC [608323]
3647	CMT intermediate	*YARS* (p.E196Q)[c]	*DNMT1, MARS*	CMTDIC [608323]
3657 (T)	CMT1	*GJB1* (IVS1-2A>G)[c]	*LRSAM1, PRX, VARS2*	CMTX1 [302800]
3660 (T)	CMT2	*MFN2* (p.V160G)[c]	*SETX*	CMT2A2 [609260]
3672 (T)	CMT2	*MFN2* (p.G176S)[c] [hmz]	*LRSAM1*	CMT2A2 [609260]
4119 (T)	CMT2	*TRIM2* (p.D667A) [hmz][a]	-	CMT2R [615490]
Phenotypic re-assessment				
996	congenital hypomielinating neuropathy/ataxia	*ITPR1* (p.G2547A)[c]	*MTMR2, WNK1*	SCA29 [117360]
1038	CMT/Dejerine-Sotas	*SURF1* (p.Q196X); (p.L105Rfs*11)[c]	*PRX, SBF2, NEFL, NGF, MYH14*	Leigh syndrome/ demyelinating peripheral neuropathy [256000]
1163	hypotonia, hypomyelinating neuropathy	*ADCY6* (p.Y992C)[c] [hmz]	-	arthrogryposis multiplex congenita with axoglial defects
1522	CMT	*MYH14* (p.R941L)[b]	-	PNMHH [614369]
1566	congenital hypotonia	*IGHMBP2* (p.C496X)[b]; (p.M449Sfs*24)[c]	*NEFL, SETX, SEPT9, YARS2*	DSMA1/ SMARD1 [604320]
1581	congenital hypomyelinating neuropathy	*IGHMBP2* (p.E514K)[b], (p.A256G)[c]; (p.A398E)[c]	*SBF2, FIG4, NTRK2, FARSB*	DSMA1/ SMARD1 [604320]
1680	neuropathy, ataxia, cataracts	*ABHD12* (14kb del)[b] [hmz]	*GDAP1*, *WNK1*	PHARC [612674]
2447	progressive neuro-degenerative disorder	*SETX* (p.Q2108X)[c] [hmz]	-	AOA2/ SCAR1 [606002]
3664 (T)	CMT2	*AIMP1* (p.Q112X)[c] [hmz]	-	hypomyelinating leukodystrophy (HLD3)
3669 (T)	CMT2	*DNAJB2* (c.619-1G>A) [hmz]	*SETX, IGHMBP2*	DSMA5 [604139]
3729 (T)	CMT2	*TFG* (p.P285L)[b]	-	HMSNO [604484]
3730 (T)	CMT2	*DNAJB2* (p.F103fs)[c] [hmz]	-	DSMA5 [604139]
Potential novel candidate genes				
124	hereditary myoclonus and progressive distal muscular atrophy	*DNAJB5* (p.P15S)	*GARS, TRPV4, RARS2*	NA
1468	CMT1	*PMP2* (p.I43N)	*PRX, VARS*	NA
1631	CMT	*SPTLC3* (p.W150R)	*MED25, SH3TC2, PRX*	NA

Abbreviations are as follows: T, patient from Turkish cohort; Dx, diagnosis; HPMV, highly penetrant Mendelizing variant; NA, not applicable. Classification of "known genes, known variants" and "known genes, novel variants" refers to the HPMV.

[a]Previously published (Pehlivan et al., 2015).

[b]Known mutation.

[c]Novel mutation in known gene.

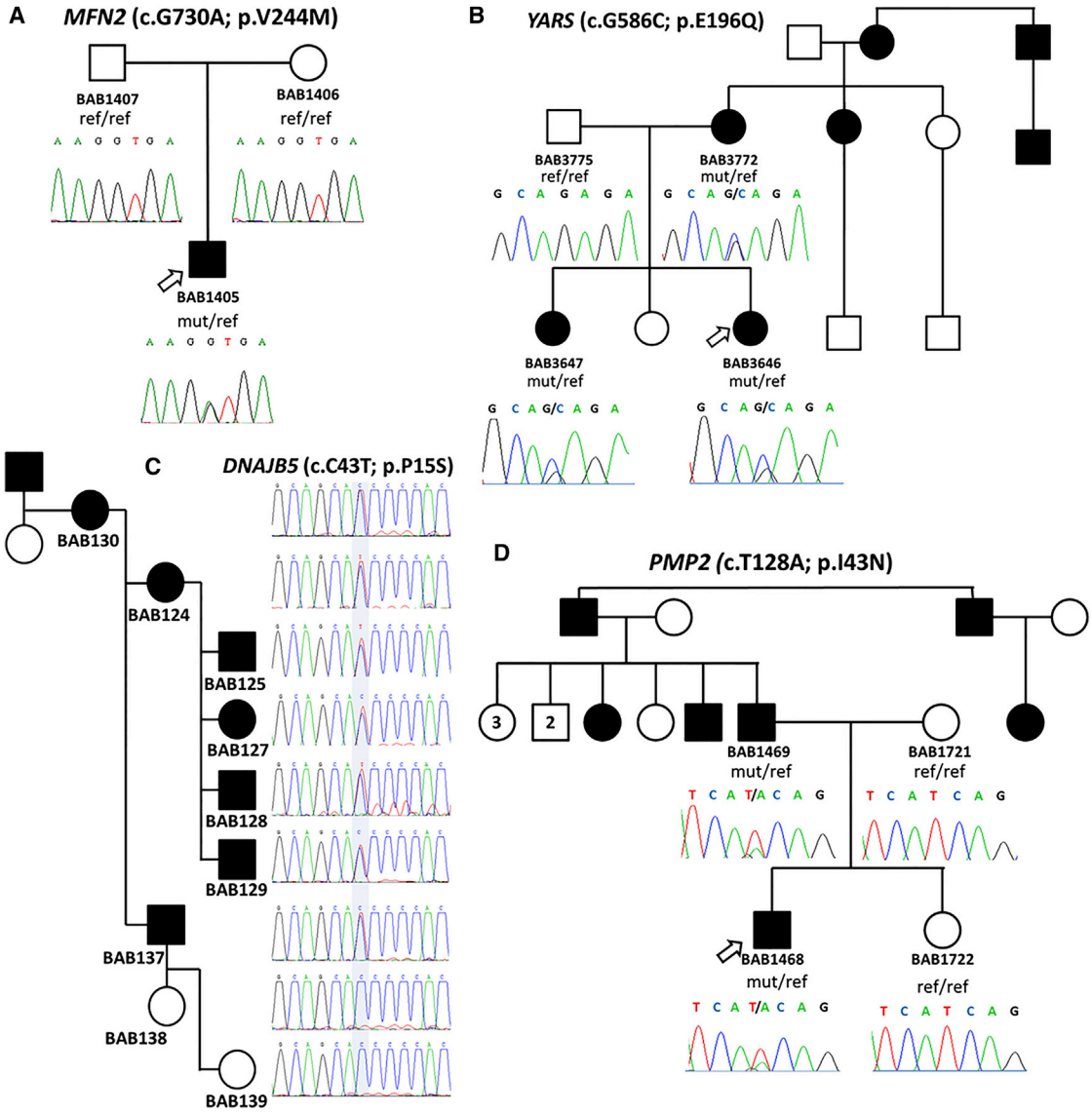

Figure 1. Pedigrees of CMT/Neuropathy Patients and Segregation of Causative Mutations

(A) Pedigree showing de novo occurrence of the known p.V244M *MFN2* mutation in proband.

(B) Dominant pedigree of a dominant intermediate form of CMT and segregation of the identified novel variant p.E196Q in *YARS*. Mutation was inherited to the affected proband and affected sister from the affected mother.

(C) Pedigree of a dominantly inherited myopathy-neuropathy phenotype in a family with multiple affected individuals where a novel variant in *DNAJB5* (p.P15S) was identified.

(D) Pedigree of a dominant form of CMT and segregation of the mutation in candidate gene *PMP2* (p.I43N). The affected proband inherited the mutation from his affected father, while both the unaffected mother and sister do not carry the mutation.

RESULTS

Known Alleles in Known Neuropathy Genes

We identified known disease-causing alleles, both single nucleotide variants (SNV) and exonic deletion CNV, in six of the 37 index patients (see Supplemental Information for detailed clinical information). Two represented phenotypic expansions of CMT2 caused by mutations in *MFN2* (Figure 1A), where the clinical presentation made screening for *MFN2* unlikely. One family showed two separate segregating causes of CMT (Verny et al., 2004), one X-linked and the other caused by compound heterozygous mutations in *MED25*. A novel, likely disease-causing allele was found in *trans* with the only known disease-causing allele in this gene (Leal et al., 2001, 2009). In a proband with autosomal dominant neurosensory deafness and axonal neuropathy, we found a recently reported mutation in *MYH14* (Choi et al., 2011). Finally, in a consanguineous family, we detected a 14-kb homozygous deletion CNV encompassing exon 1 of *ABHD12* segregating with the complex neuropathy phenotype observed in the proband and affected siblings (Fiskerstrand

et al., 2010) (Figure S2). An additional homozygous *GDAP1* novel variant was also identified in some affected individuals of this family posing the possibility of an additive contribution from intragenic deletion CNV plus SNV variation.

Novel Alleles in Known Neuropathy Genes

Rare non-synonymous, frameshifting, or splicing variants were identified in known CMT/neuropathy disease genes, illustrating the complexity that can underscore "simple" Mendelian conditions (see Supplemental Information for detailed clinical information). We identified a patient with mutations in both *MFN2* and *GDAP1*, both of which are involved in mitochondrial dynamics. Concurrent mutations in these genes have been reported, suggesting the possibility of epistasis or modifying effects (Cassereau et al., 2011; Vital et al., 2012). In a family with three generations affected by autosomal dominant intermediate CMT, we sequenced two individuals and identified a novel variant in *YARS* affecting a residue previously reported to be mutated in disease (Jordanova et al., 2006) (Figure 1B). Functional analyses revealed that the identified *YARS* allele is a functional hypomorph, unable to complement fully deletion of the endogenous yeast gene, *TYS1*, in growth complementation assays (Figure S3), supporting a pathogenic role for this mutation in CMT. A male patient with Sotos syndrome (MIM #117550) due to *NSD1* deletion plus clinical neuropathy was found to carry several predicted deleterious variants in different CMT genes in addition to a novel potentially pathogenic variant in the X-linked *AIFM1* gene (Rinaldi et al., 2012). Compound heterozygous truncating mutations in *SURF1* were identified in a proband with demyelinating CMT. Loss-of-function mutations in *SURF1* were recently described in patients with autosomal recessive severe demyelinating neuropathy of childhood onset (Echaniz-Laguna et al., 2013), consistent with this patient's clinical and molecular findings.

Genetic and Functional Evidence for Potential Candidate CMT Genes

We identified variant alleles implicating three potential new candidate neuropathy genes, *PMP2*, *SPTLC3*, and *DNAJB5*, in three different families. In a family with a clinical diagnosis of autosomal dominant demyelinating CMT1 neuropathy, we found a candidate missense variant in myelin protein P2, *PMP2* (c.T128A; p.I43N) as the most likely disease-causing variant. We confirmed this variant in the proband and his affected father, and its absence in both unaffected mother and sister (Figure 1D). PMP2 is a major stabilizing component of the myelin sheath that insulates the axons in the PNS (Majava et al., 2010) but to date has not been associated with any genetic peripheral neuropathy. *PMP2* is predominantly expressed in myelinating Schwann cells, with specific expression in sciatic nerve endoneurium and dorsal root ganglia (Zenker et al., 2014). Homozygous knockout ($Pmp2^{-/-}$) mice have significantly reduced temporal motor nerve conduction velocities, although no major structural changes in the myelin sheath and peripheral nerves were observed (Zenker et al., 2014).

In vivo modeling experiments interrogated the potential impact of *PMP2* loss of function and of this specific novel variant. Two orthologs exist in zebrafish; suppression of either using morpholino (MO) knockdown led to a motor neuron phenotype, including failure of the motor neuron axons to extend from the notochord, as well as pathfinding errors where the axons failed to innervate the myotomes appropriately (Figures 2A and 2B). These phenotypes could be rescued by co-injection of the MO with wild-type human *PMP2*; however, contrary to wild-type, human mRNA carrying the variant identified in our proband failed to restore the MO-induced phenotype (Figures 2A–2E). Upon overexpression, wild-type human mRNA induced a phenotype similar to the one observed with MO alone in >50% of injected embryos, suggestive of a dosage-sensitive transcript, similar to *PMP22*. Overexpression of human mutant (p.I43N) *PMP2* mRNA exacerbated the phenotype significantly (~20% increase; $p = 0.0003$ Figures 2E and 2F); consistent with a dominant-negative mechanism of pathogenesis for this allele.

Of note, antibodies against PMP2 fragments were identified initially in experimental allergic neuritis, an autoimmune peripheral neuropathy in animals like rats and rabbits, and a model for Guillain-Barre syndrome (GBS) (Ishaque et al., 1981, 1982). One of the main characteristics of GBS is the autoimmune attack to the peripheral nerves' myelin sheath causing demyelination. Antibodies against myelin protein zero (*MPZ*, P0) and most significantly to myelin protein 2 (*PMP2*, P2) have been detected in patients with GBS and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) characterized by primary demyelination and lymphocytic infiltration of the peripheral nerve (Inglis et al., 2007). Thus, discovery of this mutation in a CMT patient suggests a potential mechanistic link between auto-immune neuropathy and inherited neuropathy.

We identified a novel variant in *SPTLC3* (c.T448C; p.W150R) changing a highly conserved residue and predicted to be damaging by bioinformatic algorithms in a patient; no parental samples were available. The proband presented with neuropathy with a marked sensory but no apparent autonomic involvement. SPTLC3 is the third subunit of the serine palmitoyltransferase enzyme (SPT) involved in the de novo biosynthesis of sphingolipids (Hornemann et al., 2009). Heterozygous mutations in subunit 1 of SPT, *SPTLC1*, were first identified as the cause of hereditary sensory and autonomic neuropathy type 1A (HSAN1A; MIM #162400) (Dawkins et al., 2001). Both genes encoding the additional subunits of SPT, *SPTLC2* and *SPTLC3*, were screened for mutations in a cohort of typical HSAN patients. Heterozygous missense mutations were identified in *SPTLC2* in a fraction of patients but no mutations were found in *SPTLC3* (Rotthier et al., 2010). Consistent with a neuropathy "disease gene," suppression of the *sptlc3* ortholog in zebrafish embryos showed motor neuron axon defects that phenocopied suppression of other known CMT genes (Figures 2H and 2I). The specific phenotype could be rescued by co-injection with *SPTLC3* wild-type human mRNA (Figures 2H–2L). Injection of human mRNA carrying the variant identified in the proband was unable to rescue the phenotype, supporting the contention that the missense variant represents a hypomorphic or possible loss-of-function allele (Figures 2H–2L).

In a large family with an inheritance pattern consistent with an autosomal dominant myopathy/neuropathy, we identified ten shared variants in three affected individuals, of which nine did not segregate with the disease. A novel variant in *DNAJB5* (c.C43T; p.P15S) was the only rare variant that co-segregated

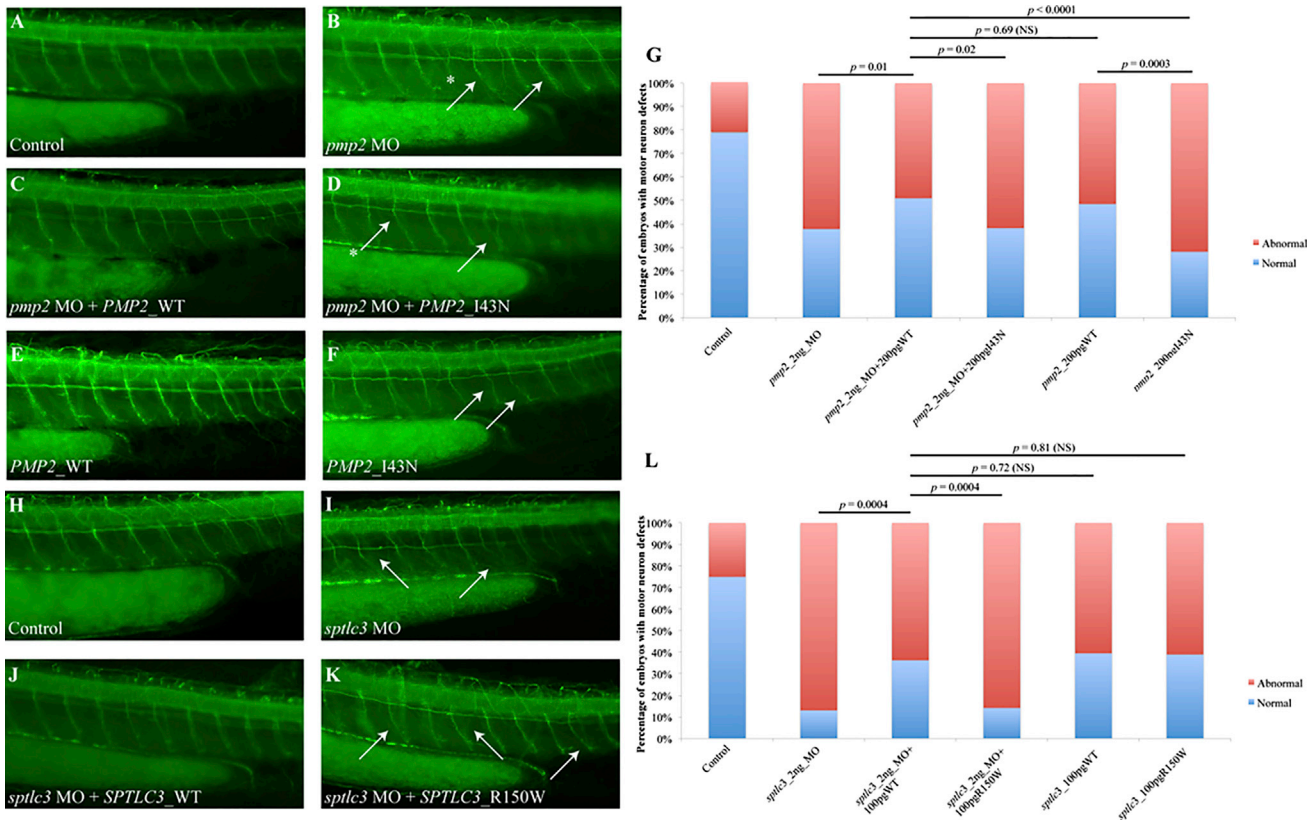

Figure 2. Suppression of pmp2 and sptlc3 in Zebrafish Causes Defects in Motor Axon Pathfinding and Outgrowth

(A–F) Lateral views of a control embryo, an embryo injected with *pmp2* morpholino (MO) and embryos injected with pmp2 MO+PMP2_WT and pmp2 MO+PMP2_I43N, PMP2_WT and PMP2_I43N cocktails, respectively, at 2 dpf (days post fertilization). Controls showed even spacing and normal branching of the motor neuron axons (A). In the pmp2 MO-injected embryos, the spacing of neuronal axons is perturbed by exiting the periphery but failing to extend (asterisks) or presenting pathfinding errors (arrows; B). Co-injection of pmp2 MO with human PMP2_WT resulted in restoration of the normal neuronal phenotype (C), but PMP2_I43N did not (D). Overexpression of human PMP2_WT causes mild pathfinding errors (E), suggesting dose sensitivity for *PMP2*. However, the human PMP2 mutant p.I43N was significantly more severe than PMP2_WT when overexpressed (F) and had similar effects to suppression of *pmp2* by MO knockdown.

(G) Percentage of normal versus abnormal embryos under the conditions being evaluated above.

(H–K) Wild-type embryos (H) and *sptlc3* morphants (I) in which secondary axons fail to migrate appropriately (white arrows). The phenotype induced by suppression of *sptlc3* could be rescued by co-injection with SPTLC3_WT (J) but not SPTLC3_R150W (K).

(L) Quantification of normal embryos versus embryos with motor neuron axon defects. For statistical analyses, χ^2 tests were performed.

with the phenotype (Figure 1C). This rare variant was observed in four other independent individuals in our exome database of ~3,000 individuals; however, no phenotypic information is available for these individuals. The variant is also present in the heterozygous state in a single individual in the Exome Aggregation Consortium (ExAC) compiled data set (MAF = 0.00004858). This *DNAJB5* variant affects a highly conserved amino acid in the DnaJ domain of the protein. A homozygous mutation in *DNAJB2* was identified in a large family segregating recessive distal hereditary motor neuropathy of early adulthood onset (Blumen et al., 2012). Mutations in *DNAJB6* have also been implicated in autosomal dominant myopathy (Harms et al., 2012; Sarparanta et al., 2012) and have a dominant-negative toxic effect increasing the stability of the cytoplasmic form of the protein and interfering with its chaperone function (Sarparanta et al., 2012). These three genes encode members of the HSP40/DNAJ family of molecular co-chaperones, which protect proteins from irreversible aggregation during protein synthesis or molecular stress. Functional testing of this gene by MO knockdown in zebrafish showed abnormal peripheral nerve axonal architecture supporting a role of this gene in peripheral nerve pathophysiology but had no apparent effect on muscle architecture (Figure S4). We propose *DNAJB5* as a potential candidate for myopathy/neuropathy based on its relationship with previously reported genes involved in similar phenotypes; *HSPB8* (HSP27) and *HSPB1* (HSP22) are known genes associated with peripheral neuropathy (Evgrafov et al., 2004; Irobi et al., 2004).

Rare Variant Contributions to Phenotypic Manifestations: Evidence for a Mutation Burden

WES of neuropathy patients often identified more than one rare variant in a neuropathy gene within a given personal genome (Tables 1 and S2). As described above, we identified the predominant highly penetrant Mendelizing variants (HPMV) in multiple patients, as evidenced by co-segregation with disease or de novo appearance in sporadic neuropathy. However, we

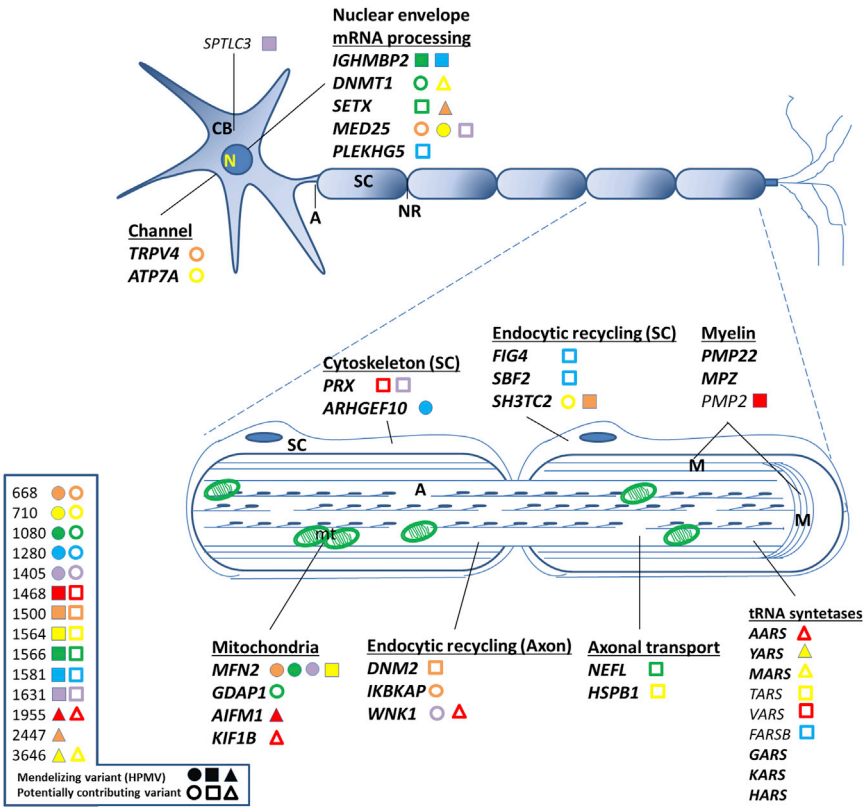

Figure 3. Neuron Schematic of the Localization or Site of Action of the Main CMT/Neuropathy Gene Products

Legend on left shows patient identifier numbers and causative and possibly contributing mutations identified by WES. Full shapes correspond to rare presumed causative mutations deemed Highly Penetrant Mendelizing Variants (HPMVs), while empty shapes correspond to rare variants that may be contributing to the mutation burden in neuropathy patients. Each personal genome is distinguished by a unique color/shape. In boldface are some of the canonical CMT genes. Abbreviations: CB, neuron cell body; A, axon; SC, Schwann cell; NR, node of Ranvier; mt, mitochondria; M, myelin.

also identified potential contributing or modifying rare variants in other neuropathy-associated genes (Figure 3). These latter rare variants are not likely the mutations predominantly responsible for trait manifestation because they are inherited from an unaffected parent or do not conform to Mendelian expectations (i.e., exceptions to co-segregation with neuropathy in the family). For example, we observed a higher than expected heterozygous carrier frequency of the reported *MED25* (p.A335V) mutation in our cohort (10% of patients; MAF = 5.0%) compared to that observed in the NHLBI ESP study sample (65/6,498 individuals; MAF = 0.5% [p value = 0.001]), a group of 266 controls (two of 266 individuals; MAF = 0.375% [p value = 0.003]), and the ARIC European-American (ARIC-EA) study participants (80/5,748 individuals; MAF = 0.7% [p value = 0.003]). Although in three of four cases in our patient cohort, there is no "second hit" in *MED25* to cause the CMT2B2 phenotype, we cannot discount the possibility of a second pathogenic non-coding variant not captured by WES or the potential contribution of this mutation in a mutational aggregation model to the overall phenotype of these patients.

Of note, we identified an average of 2.3 nonsynonymous rare variants per individual in 58 known neuropathy-associated genes in the entire patient cohort (40 samples) versus 1.3 nonsynonymous rare variants in 5748 ARIC-EA control individuals ($p < 0.0001$; Figure 4A). Cases with a definitive molecular diagnosis had an average of 2.9 variants per individual (including the HPMV), while the undetermined cases had an average of 1.8 variants per individual. After implementing a stringent filter where we subtracted the HPMV of each molecularly defined case, we still found an average of 1.8 variants in the CMT cohort versus 1.3 in controls ($p = 0.007$), similar to the average of mutations in only the cases without a yet-definitive HPMV (Figure S5). These data suggest that the mutation burden in CMT genes remains the same between patients with a known versus unknown HPMV and is significantly greater than the background load in unaffected controls.

As a further test of this mutational burden observation, we calculated repeatedly the average number of rare, nonsynonymous variants in the 58 neuropathy genes in 40 randomly selected individuals from the BHCMG_EU sample set compared to the 5748 ARIC_EA controls. Upon conclusion of 100 resamplings (with replacement), we only found three instances in which the p value was lower than the p value observed in our original US CMT (subtracting the HPMV) versus ARIC_EA analysis. These data reinforce the notion that the background mutation load in these 58 neuropathy genes is specific to the population of neuropathy patients.

To further investigate our observation of neuropathy gene mutation burden in neuropathy patients, we analyzed WES data from an independent cohort of 32 patients (30 families) from Turkey with a clinical diagnosis of CMT. When compared to population-matched unrelated Turkish controls, the Turkish neuropathy cohort had a mutation burden of 2.1 versus 1.6 ($p = 0.013$) nonsynonymous rare variants per individual, lending further credence to the mutation burden hypothesis (Figure 4B; Figure S5). The smaller difference in the number of rare variants per individual may also reflect a greater number of private variants in the Turkish population (particularly recessive alleles) or the contribution of consanguinity in this population.

Functional Testing of the Mutation Burden Hypothesis

We hypothesized that the "mutation burden" observed in the CMT cohorts would be reflected in the functional consequences of CMT gene knockdown, and combinations thereof, in a zebrafish model. This functional assay evaluated the integrity and innervation of motor neuron axons along the body axis (Figure 5). A subset of genes was tested for potential genetic interactions

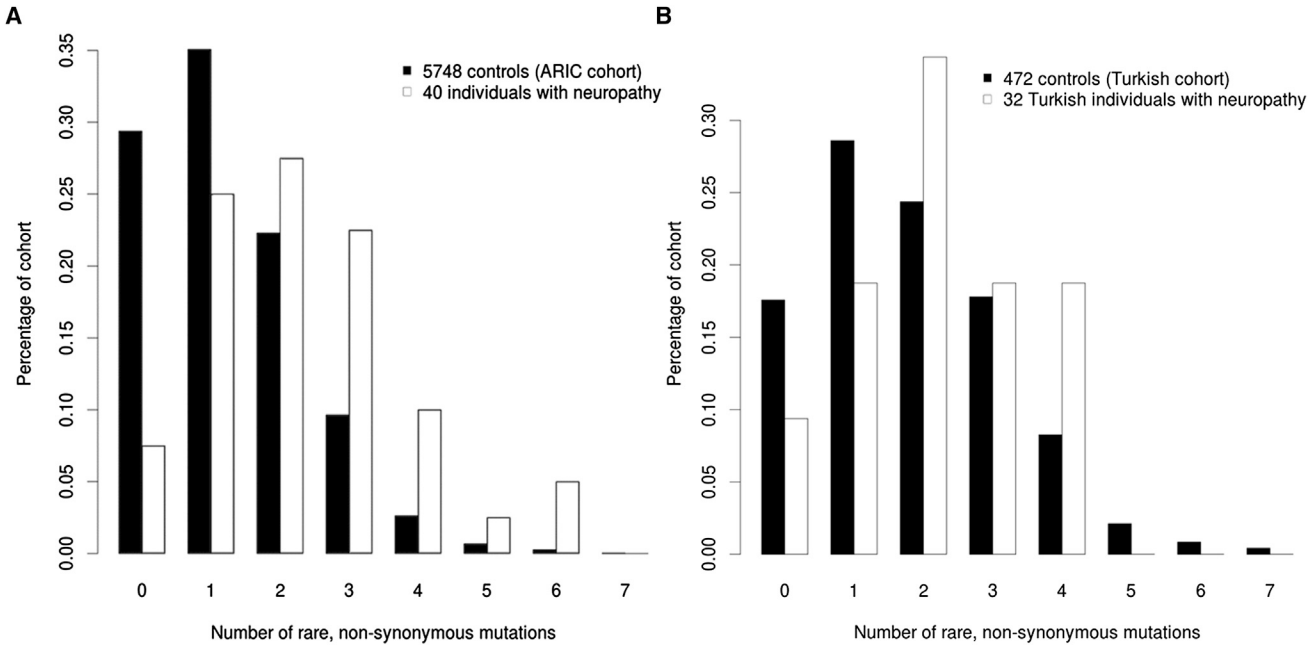

Figure 4. Rare Variant Distribution in Studied Individuals Suggests High Carrier Frequency for Rare Alleles in Neuropathy Genes in Exome Sequenced Neuropathy Cohort

(A) Distribution of rare variants in neuropathy associated genes in 40 patients of neuropathy cohort (white bars) shows a tendency toward harboring more rare variants per individual exome as compared to a different extended cohort of 5,748 Europeans from the ARIC-EA study (black bars) observed to have a tendency toward zero or one rare variants in neuropathy genes.

(B) A similar trend is observed in an ethnically different cohort of patients with neuropathy from Turkey (white bars) as compared to ethnically matched Turkish control individuals without neuropathy (black bars).

and mutation burden effects on phenotype based on our initial cohort's observed mutation events. Specifically, we suppressed each of *mfn2*, *gdap1*, *abhd12*, *med25*, *hspb1*, and *wnk1* separately and in pairwise combinations of sub-effective doses and tested the functional consequences of the genetic interactions between the selected CMT genes. Consistent with our hypothesis, we observed increased severity in the phenotype of aberrant axon extension, branching, pathfinding, and morphology of peripheral neurons in our zebrafish model when we injected pairwise combinations of these genes (Figure 5). In each case, we observed likely multiplicative effects, although the magnitude of interaction was unique for each pairing. For example, sub-effective co-injection of MOs against *mfn2* and *gdap1*, which by themselves gave no phenotype at the dose tested, yielded a milder exacerbated phenotype (class I/II motor neuron pathology in 80%–100% of embryos tested), whereas co-suppression of *mfn2* and *med25* yielded 80%–100% affected embryos, with one-third of the embryos affected severely (class III/IV). These data support the prediction of genetic interaction for loss-of-function events in bona fide CMT genes. To assess the specificity of our in vivo model, we also tested for genetic interaction between *GDAP1*, a bona fide CMT driver, and three genes that have not been associated previously with peripheral neuropathy. Two of those are expressed in the CNS and cause other neuropathologies (*SIX6*: optic nerve atrophy [Carnes et al., 2014]; *RP1L1*: retinal degeneration and cerebellar disorganization [Davidson et al., 2013]), and the third is expressed ubiquitously (*ANKRD6*). We injected sub-effective doses of each of the tested genes alone and also in pairwise combinations (Figure S6). Though *RP1L1* yields a 20% increase in the percentage of embryos with abnormally formed peripheral neuronal axons when injected alone, we observed no exacerbation of the phenotype when each of those genes was suppressed in combination with *GDAP1*.

DISCUSSION

Whole-exome sequencing (WES) allows genome-wide assessment of SNV coding variation in the fraction of the human diploid genome that we can potentially interpret. However, even in genetic conditions with known associated genes, interpretation can be complicated by the presence of novel variants in more than one causative gene (Yang et al., 2013, 2014). Additionally, the contribution of variants in a multiplicity of genes for a single condition within an individual personal genome and how variation in these can contribute to or modify the phenotype has rarely been assessed.

We identified the apparent HPMV and likely primary disease driver of the neuropathy phenotype in 17/37 (45.9%) families studied and suggest a potential candidate gene for three additional families. We discovered a mutational burden of 2.3 damaging variants in CMT patients versus 1.3 in controls for the 58 neuropathy-associated genes examined ($p < 0.0001$). After a highly stringent additional filter consisting of subtraction

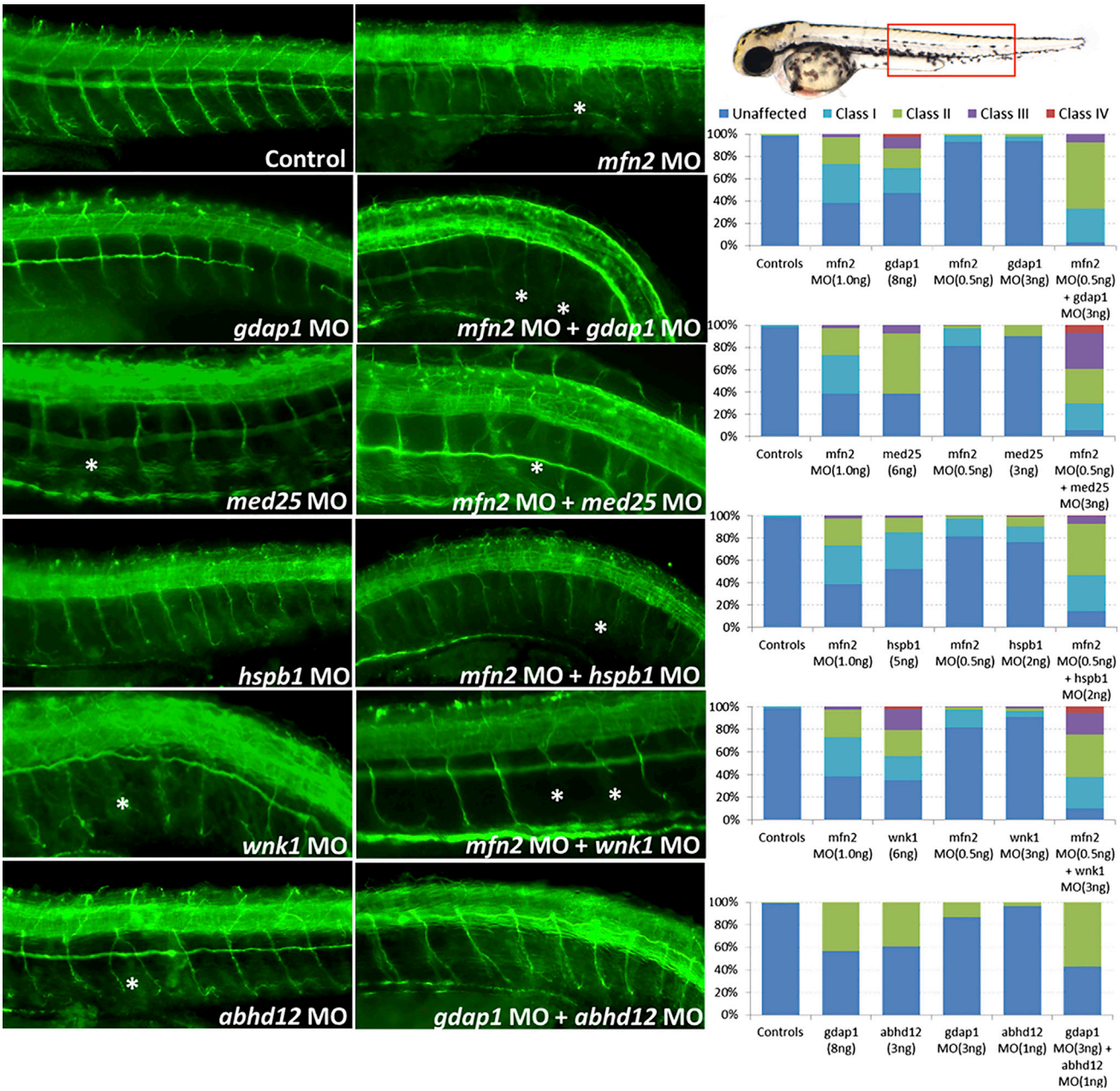

Figure 5. Functional Assessment of Mutation Burden Hypothesis in a Zebrafish Model

First and second column panels show representative images of acetylated-tubulin (α-AcTub) staining of peripheral neurons in 2-day MO knockdown, single or in pairwise combinations, zebrafish larvae. Third column panel shows qualitative assessment of morphant fish evaluated as defects in peripheral neuron axon extension, branching or pathfinding according to the scoring system developed. For pairwise combinations, sub-effective concentrations of each of the gene-specific MOs were injected as shown in the graphs by the number of abnormal larvae in each category. However, when injected together increased severity in the phenotype was observed for all the pairwise combinations, suggesting in vivo epistatic effects between these pairs of genes as observed in the α-AcTub fluorescence images and quantified in the graphs. Asterisks highlight some evidently affected axons. The scoring system used for assessing PNS defects in zebrafish was developed ad hoc and implemented here in order to best reflect the observations resulting from our experiments. Class I category refers to single axon defects; class II category refers to two or more axons exhibiting defects with the presence of some normal axons; class III category refers to generalized axonal defects; class IV category refers to complete absence of axonal extension.

of the HPMV, neuropathy patients carry a mutation burden consisting of an average of 1.8 rare variants in neuropathy-related genes, as compared to an average of 1.3 rare variants in a control population ($p = 0.007$). A mutation burden ($p = 0.013$) was replicated in a second, ethnically distinct CMT cohort in comparison to ethnically matched controls. This mutation burden may

well influence the phenotype, contributing to the clinical heterogeneity and the spectrum of severity observed in the disease (Haldane, 1941). We explored this hypothesis in vivo examining phenotypic consequences of genetic interaction between select pairs of neuropathy genes. We observed increased severity of the phenotype in zebrafish consistent with potential additive and positive genetic interactions between neuropathy genes.

Our cohort has an intrinsic bias since individuals had previous extensive clinical and molecular screening for disease-causing variation in the most common CMT genes prior to consideration for WES. As anticipated, we found a low frequency of known mutations as these samples were previously screened for such variants. We found variants in known CMT or neuropathy genes in 17 cases, including one (*MFN2*) showing phenotypic expansion in a CMT1 family. By expanding our candidate list to include additional neuropathy-associated genes, we achieved a 45.9% (17/37) mutation detection rate. Furthermore, we identified likely candidate genes *PMP2*, *SPTLC3*, and *DNAJB5* in an additional three families potentially providing molecular insights into 20/37 (54.1%) of the families. We also provide functional evidence for the pathogenicity of the identified variants in *PMP2* and *SPTLC3* (Figure 2) and the effect of *dnajb5* suppression on motor neurons (Figure S4). However, conclusive proof for these genes representing bona fide "neuropathy disease genes" will require the identification of pathogenic variants in additional patients.

Analysis of the WES data from this neuropathy cohort illustrates limitations of clinical phenotyping. Detailed phenotypic information is required for correlating potential disease-causing variants to the clinical phenotype of patients. As illustrated in 12 of the study subjects, eight from the initial cohort and four from the Turkish cohort originally referred for a presumptive clinical diagnosis of CMT, after a molecular diagnosis by WES and upon retrospective re-evaluation of clinical records, the broader spectrum of additional clinical features suggested other disorders associated with neuropathy. Moreover, these further refined phenotypes were consistent with the molecular findings from WES in each of the identified genes (Table S3). The phenotype driven paradigm for clinical diagnosis is limited by the (1) presentation of the patient at the given time, (2) individual examiner, and (3) underlying assumption of a singular unifying diagnosis; the latter potentially not applicable to either a mutation aggregation model or a mutation burden hypothesis.

In 29/40 (72.5%) patients, we identified additional "carrier status" mutations in other CMT or neuropathy-associated genes besides the apparent HPMV (Tables 1 and S2). These additional variants might contribute to the variability of expression of the clinical phenotype (Haldane, 1941). Furthermore, in the cases where specific HPMVs were not identified, novel loci potentially wait to be recognized as main disease drivers (Table S4), but the mutation burden may still contribute to variable expressivity of the neuropathy phenotype. It is possible that mutation burden and combinatorial effects of rare variants in genes that interact genetically in the same biological pathways, such as those of tRNA biogenesis, endocytic recycling or mitochondrial dynamics, modify the phenotype due to synergistic (exemplified by *MFN2* and *GDAP1* co-occurring mutations in the same patient) or counteracting effects (Klassen et al., 2011; Davis and Katsanis, 2012). Alternatively, or additionally, the cumulative mutation burden in genes dispersed across various biological pathways or "networks" might interplay to destabilize or compensate the system and thus modulate the penetrance and/or expressivity of the overall phenotype. Although robust, the capacity of biological networks to buffer perturbations may be limited if various mutational events are coincident in a personal genome. Studies of the human disease network (Goh et al., 2007; Hidalgo et al., 2009) at the genomic scale will likely contribute to our understanding of both disease and homeostatic states in human biology.

Genome-wide approaches have shown that rare variants are more common than previously thought (Coventry et al., 2010; Marth et al., 2011), a robust observation for both SNV- and CNV-disease-associated alleles (Boone et al., 2013). The overall phenotype of a given individual may to a greater extent represent contribution of either de novo or more recent and private mutational events with bigger effects on the whole system function, the "driver" genes that occurred in the recent ancestors of the individual or clan (Lupski et al., 2011), rather than more distributed common variants shared in a population or throughout several populations. This mutation burden hypothesis and its role in clan genomics is further illustrated in CMT1A duplication families wherein a phenotypic outlier in the family is recognized when the duplication becomes a triplication (Liu et al., 2014) or a CMT1A duplication is "homozygosed" in a severe neuropathy patient born to heterozygous affected parents (Lupski et al., 1991).

Interestingly, within peripheral neuropathies, several disorders once thought to be mostly caused by environmental factors, have been subsequently shown to have a genetic susceptibility component. A key example is provided by CNV at the *PMP22* locus. The reciprocal to the CMT1A duplication, deletion of 17p11.2, causes hereditary neuropathy with liability to pressure palsies (HNPP) (Chance et al., 1993). Trait manifestation is usually associated with an environmental insult, trauma to a specific nerve and often those that come anatomically close to the surface (e.g., the ulnar nerve responsible for the 'funny bone' phenomena of numbness and tingling upon hitting the elbow). Locus-specific molecular studies revealed the majority of individuals that carry the HNPP deletion go undiagnosed (Turner et al., 2008) due to phenotypic variability or lack of clinical symptoms (Kumar et al., 1999). However, association of the deletion carrier status with susceptibility to developing carpal tunnel syndrome (CTS) has been documented (Cruz-Martinez and Arpa, 1998; Potocki et al., 1999; Del Colle et al., 2003). Additionally, 24 of 51 patients diagnosed with multifocal neuropathies, not considered a genetic disease, were found to carry the HNPP deletion. Moreover, 37% of mutation positive subjects had no family history of neuropathy (Tyson et al., 1996). Consequently, haploinsufficiency of the dosage-sensitive *PMP22* gene, either by HNPP deletion (CNV) or loss-of-function point mutations (Nicholson et al., 1994; Shy et al., 2006), has been associated with susceptibility to milder forms of neuropathy. Furthermore, haploinsufficiency of the CMT *SH3TC2* gene can also confer subclinical neuropathy phenotypes in heterozygous carriers, including subclinical axonopathy and median nerve mononeuropathy associated with susceptibility to CTS (Lupski et al., 2010).

From this perspective, our identification of a *PMP2* variant, a gene whose product has been linked to experimental autoimmune neuropathy and both Guillain-Barre syndrome (GBS) and

chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), in one family suggests a potential genetic susceptibility to autoimmune neuropathy. Haploinsufficiency of other CMT or neuropathy genes can also contribute to susceptibility to multifactorial neuropathies. Moreover, a recent study to survey possible underlying genetic contribution to developing chemotherapy-induced peripheral neuropathy (CIPN) due to allelic variability in known CMT genes identified an association of *PRX* heterozygous variants in individuals that developed CIPN versus controls similarly exposed (Beutler et al., 2014). Additionally, three common SNPs in *ARHGEF10* were also associated with different outcomes of protection and susceptibility to CIPN in the same cohort (Beutler et al., 2014). These findings support and highlight one of the main hypotheses from the present study; the mutation burden of carrier status, for neuropathy-associated rare variant recessive alleles, in clinically unaffected individuals can poise the organism to develop other types of complex neuropathies later in life upon gene-environment interactions (GxE). External insults, chemical or mechanical; other pathologic processes like diabetes or infection; or aging with concomitant prolonged exposures and/or reduced biological function of cells (e.g., SSBR, gene transcription, protein processing and folding, etc.) or functional units like the neuron can be the critical factor for the system to express the disease later in life. This might also be true for other traits thought to be complex and having a major environmental influence with a reduced genetic component that have been elusive to other approaches. Rather than single-locus strong associations across populations, each individual with such a given complex disorder can carry a handful of rare/private variants in a variety of genes in their personal genome that are important for the development of the disease process and that through an oligogenic model confer susceptibility to the individual to develop the disorder upon additional factors such as diet, exposures, aging, etc.

In summary, our studies of rare genomic variants in neuropathy identify known pathogenic alleles, novel variants in known disease genes, and further document phenotypic expansion for disease gene traits. We identified three potential novel candidate neuropathy "disease genes" as supported by both genetic and functional studies. Moreover, we provide evidence that genome-wide studies and molecular diagnosis can further assist interpretation of a clinically based differential diagnosis. Of note, systematic analyses of genes implicated in neuropathy reveal a mutation burden in patients compared with unaffected control populations and zebrafish model organism studies show gene interactions for genes implicated by mutation burden in individual families. This mutation burden is consistent with the concept of clan genomics (Lupski et al., 2011) contributing significantly to both Mendelian and common/complex disease trait manifestation.

EXPERIMENTAL PROCEDURES

Samples

We performed WES through the Baylor-Hopkins Center for Mendelian Genomics (BHCMG). Written informed consent from all participating subjects was obtained for DNA and genetic analyses though a Baylor College of Medicine Institutional Review Board approved protocol and also approved by the BHCMG ELSI committee for inclusion into the BHCMG sequencing project. Some of these samples had been collected and stored over decades; thus, DNA of parents or other family members was not always available for additional testing and co-segregation analyses.

Exome Sequencing

We performed whole-exome next-generation sequencing according to previously published methods (Lupski et al., 2013; see Supplemental Experimental Procedures for details), producing an average of 9.25 Gb of raw data per exome and achieving ~93.5× average depth of coverage (median coverage = 97×) per sample with >90% of the captured bases covered at 20× (Table S5). Variant data generated will be released and deposited into the NCBI database of Genotypes and Phenotypes (dbGaP: http://www.ncbi.nlm.nih.gov/gap) as part of the Centers for Mendelian Genomics research initiative.

Variant Annotation Pipeline

Variant calling from the aligned BAM files was performed using the ATLAS (Shen et al., 2010) and SAMtools suites (Li et al., 2009). Annotation was performed using Sacbe, an in-house developed annotation pipeline (Gonzaga-Jauregui et al., 2013) based on ANNOVAR (Wang et al., 2010) and custom scripts (see Supplemental Experimental Procedures for details).

Data Analysis

We performed an initial analysis focusing on a list of 74 CMT and other neuropathy-associated genes (Table S6). Additionally, we interrogated a list of candidate CMT genes (Table S7) based on first degree interactors of known CMT genes and performed a second pass analysis in those cases where we did not identify candidate mutations in CMT genes.

The number of rare (i.e., minor allele frequency of $\leq$1% in TGP, NHLBI ESP, and the European subset of NHLBI ESP) nonsynonymous variants in 58 well-established CMT genes (Table S8) was computed for each sample of the neuropathy cohort and for 5,748 Europeans from the ARIC (Atherosclerosis Risk in Communities study) cohort, a large population-based study of cardiovascular disease and its risk factors. The average number of rare nonsynonymous variants was then compared between the neuropathy and ARIC study samples using a non-parametric Mann-Whitney-Wilcoxon test. A permutation procedure with 100,000 iterations was performed to determine statistical significance. For the second CMT cohort of Turkish descent, a set of 472 Turkish controls was used that was sequenced and analyzed using identical protocols, platforms, and standards to those of the cases.

Functional Experiments

See Supplemental Experimental Procedures for details.

ACCESSION NUMBERS

The accession numbers for the exomes reported in this manuscript consented for data deposition into dbGaP are SAMN03361200, SAMN03361023, SAMN03361097, SAMN03361077, SAMN03361019, SAMN03360995, SAMN03361140, SAMN03361181, SAMN03361070, SAMN03361130, SAMN03361182, SAMN03361149, SAMN03361075, SAMN03361073, SAMN03361017, SAMN03361098, SAMN03361038, SAMN03361007, SAMN03361168, SAMN03361050, SAMN03361083, SAMN03361138.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.023.

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C.G.-J. declares no conflict of interest associated with the work presented in this manuscript; she is currently an employee of Regeneron Pharmaceuticals Inc. M.N.B. declares no conflict of interest associated with the work presented in this manuscript; he is the founder of Codified Genomics, LLC, a genomic interpretation company. J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals, has stock options in Lasergen, Inc., is a member of the Scientific Advisory Board of Baylor Miraca Genetics Laboratories (BMGL), and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases and bacterial genomic fingerprinting. R.A.G is currently interim CSO of BMGL. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from the chromosomal microarray analysis (CMA) and clinical exome sequencing offered in the Baylor Miraca Genetics Laboratory (BMGL: http://www.bmgl.com/BMGL). All other authors have no conflicts of interest to declare associated with the work presented in this manuscript.

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